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TITLE: Twist Maintains Stemness in Latent Breast Cancer Metastases Through a Novel Non DNA-Binding Function Targetable With Small Molecules

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14. ABSTRACT The Twist induced EMT program in a primary tumor is required for proliferation, migration, invasion and colonization of distant organs. Once cells are disseminated, a genetically and biochemically separate domain of Twist: the WR domain functions to maintain the metastatic tumor cells in a quiescent (possibly pluripotent) state by rendering the cells resistant to differentiation inducing signals, similar to what happens during development. The COOH-terminal Twist WR domain is required for binding directly to lineage specific transcription factors and inhibiting their function. The goals of this project are to define how Twist and the WR domain function to maintain the undifferentiated state in these latent disseminated cells. Progress in this first year of support includes: 1. Definition of the spectrum of proteins bound by to endogenous, cell derived Twist in a breast cancer cell line undergoing EMT. 2. Reconstitution of this complex with purified recombinant Twist -E47-Snail and Sox9 proteins. Consistent with our original hypothesis and AIMS, the complex contains DNA binding proteins which do not bind their sites when bound to the WR domain. Our Goals and SOW remain unchanged.					
15. SUBJECT TERMS Breast Cancer, Metastasis, EMT, differentiation, stem cell, Twist, RUNX, Gene Regulation					
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- 1. INTRODUCTION:** We hypothesized that the Twist WR domain is required for establishment and maintenance of the stemness phenotype that is induced in breast cancer cells as a result of the EMT program and that the WR domain functions as a protein-protein interface that binds to and inhibits lineage specific differentiation transcription factors, thereby maintaining the stemness state. The overall Objective is to define the mechanism by which the WR maintains stemness so that strategies can be identified to manipulate the Twist-EMT induced stem cell state in disseminated tumor cells by for instance, inducing differentiation or by enforcing long term permanent stemness resulting in therapeutic benefit.
- 2. KEYWORDS:** EMT, mesenchyme, differentiation, gene regulation, twist, E47, DNA binding protein, transcription, crystal structure, stem cell, stemness, metastasis, dissemination, dormancy.

3. ACCOMPLISHMENTS:
What were the major goals of the project?

SOW Major Goals - % completed to date

Specific Aim 1: Define the role of candidate proteins bound by Twist WR in the induction of EMT and maintenance of the stemness phenotype, and search for novel proteins bound by WR in Twist induced stem cell populations.

90% completed

Specific Aim 2: Solve the structure of the WR domain when bound to the RUNX2 to determine how this interaction inhibits the binding of RUNX2 to DNA as a model for how Twist accomplishes this for other targets.

10% completed

Specific Aim 3: Define the role of the Twist bHLH and WR domains in induction of EMT, generation of cells with a stem cell phenotype and the maintenance of this phenotype using site directed mutagenesis of each domain in concert in vitro and in vivo cell models of EMT and stem cell generation.

5% completed

What was accomplished under these goals?

Accomplishments; By AIM in the SOW:

Specific Aim 1:

Define the role of candidate proteins bound by Twist WR in the induction of EMT and maintenance of the stemness phenotype, and search for novel proteins bound by WR in Twist induced stem cell populations.

Conserved amino acids in the WR domain has been subjected to site directed mutagenesis: most of the changes are to alanine and expected to change protein-protein interactions attributed to that AA/region of the protein. Each of these proteins have been expressed, purified, and used for DNA binding assays as shown in Figure 1 (also shown in last yearly report: presented here just for introduction and clarity). Although some of the mutant can be expressed at a detectable level, we were successful in purifying

wildtype and 23 mutant proteins (Figure 1). As can be seen, a number of these single AA substitutions alter the ability to form a tetramer on DNA. These mutant proteins/genes were also tested for induction of EMT in SUM1315 and HMLE cells and for the ability to bind to Runx1 and inhibit lineage specific differentiation. Thus, AIM 1 supplied a large spectrum of site directed mutants which were used to explore the correlation between: 1. WR domain function, 3. tetramerization, 4. induction of EMT and inhibition of a lineage specific transcription factor (Runx2). Using this beautiful set of site-directed mutations in Twist-WR, we expected to find a correlation between these functions (listed 1-4, above). However, despite an exhaustive analysis of both wild type and mutant Twist-WR proteins with respect to these functions, we could not do so.

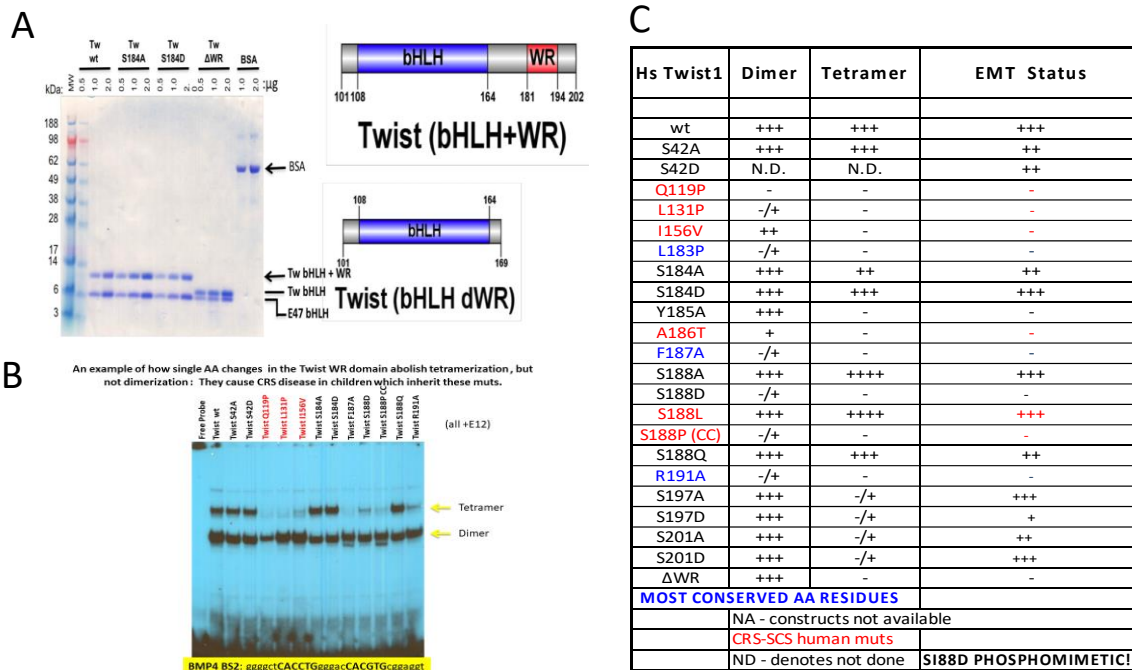
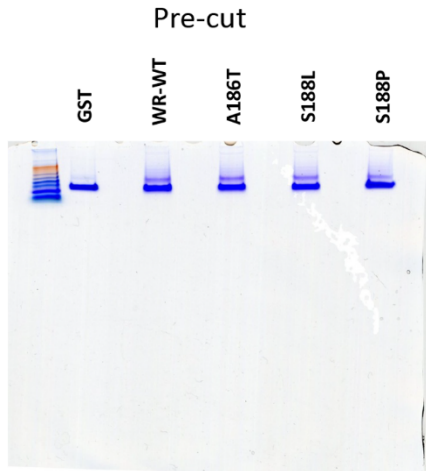


Figure 1. A. Biochemical purification of TWIST and its WR domain deletion mutant. B. Mutations in Twist can affect dimerization, tetramerization and DNA binding of the protein. Electrophoresis Motility Shift Assays (EMSAs) have been utilized with P^{32} γ -ATP labelled DNA probes to show the impact on binding that different mutations have on the double E-box transcriptional activation domain of a Twist1 target gene for EMT, BMP4. These mutations are derived from both developmental disease relevant mutations in patients and computational methods in the lab. Tetramerization of Twist1 and binding partner (E12 in this case) on the double E-box regulatory elements are necessary for activation of EMT and can be clearly distinguished from the less relevant dimerization. C, Summary of DNA binding, dimerization, tetramerization, and EMT phenotype for each of the TWIST mutations generated.

Proteomic analysis of Twist WT and Mutant complexes. New data is provided showing the results of a LC/MS/MS analysis of proteins that are associated with Twist wild-type (WT) or mutant proteins defective for EMT were isolated from stable cell lines SUM1315 and analyzed for associated proteins. The top hits are listed in **Fig. 2**.



Protein names	>10-fold lower In A186T vs WT	>10-fold lower In S188L vs WT	>10-fold lower In S188P vs WT
DNA repair protein RAD51 homolog 4	*	*	*
Metaxin-2			*
Cancer-related nucleoside-triphosphatase			*
Serine/threonine-protein kinase MST4			*
Lysophospholipase-like protein 1	*		
Transmembrane and coiled-coil domain-containing protein 1			*
Transmembrane emp24 domain-containing protein 1		*	
Transportin-3	*		*
Vesicle transport protein GOT1B			*
Deubiquitinating protein VCIP135		*	

Figure 2. Proteomic analysis of Twist complexes. Proteomics Methods: Samples were run into a gel for a short distance of 0.5 cm and stained with Coomassie blue. The entire stained gel regions were excised, digested in-gel with trypsin, and analyzed by LC-MS/MS on the ThermoScientific Q Exactive Plus mass spectrometer. MS data were searched against the UniProt human database using the MaxQuant 1.5.2.8 program. False discovery rates (FDR) for protein, and peptide were set at 1%.

Apart from the technical issues related to experimental feasibilities (unable to purifying some of the mutants), AIM 1 is 90 % completed.

Specific Aim 2: Solve the structure of the WR domain when bound to the RUNX2 to determine how this interaction inhibits the binding of RUNX2 to DNA as a model for how Twist accomplishes this for other targets.

For AIM 2, we started to make the construct in order to map the interaction between Twist-WR and Snail. Because we showed beautifully co-expressed, and purified (from E.coli) Twist-WR and E47 minimal proteins which we showed are highly functional in dimerization, tetramerization and binding to a DNA target site (Fig. 1). We plan to use these protein preparations to reconstitute Runt domain DNA binding to its target site and its inhibition by Twist-WR (+E47).

AIM 2 is 10% complete.

Specific Aim3. Define the role of the Twist bHLH and WR domains in induction of EMT, generation of cells with a stem cell phenotype and the maintenance of this phenotype using site directed mutagenesis of each domain in concert in vitro and in vivo cell models of EMT and stem cell generation.

We have started to establish the hormone-inducible cell lines in preparation for functional studies.

In summary, we have made excellent progress and are roughly on time with our commitments based on the SOW. Nothing has changed.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

We demonstrated our ability in co-expressing, and purifying (from E.coli) Twist-WR and E47 minimal proteins which we showed are highly functional in dimerization, tetramerization and binding to a DNA target site. In the next reporting period we plan to solve the structure of the WR domain when bound to the RUNX2 to determine how this interaction inhibits the binding of RUNX2 to DNA as a model for how Twist accomplishes this for other targets in Aim 2. In addition, we plan to establish the hormone-inducible cell lines for functional studies as proposed in Aim 3.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

We have established a unique protein co-expression system using both tagged and un-tagged proteins, all contained in a single plasmid DNA. This method should be very useful for other investigators to try. The co-expression of soluble active Twist-E47 complex has never been accomplished by others. We are the first to do it.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

No Changes requested

Actual or anticipated problems or delays and actions or plans to resolve them

Problems of protein expression are described above in the narrative. They have been solved.

Changes that had a significant impact on expenditures

No Changes Required or anticipated.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of human subjects.

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents.

Nothing to report.

6. PRODUCTS:**Publications, conference papers, and presentations**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Name:	Dan McCracken
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	<i>Dan has cloned, expressed and purified Twist-E47 complexes and has shown the twist complex in SUM1315 cells.</i>
Funding Support:	<i>NIH T32 Cancer Biology Training grant</i>

Name:	Frank J. Rauscher III, Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Overall Supervision and Direction
Funding Support:	This award

Name:	Hongzhuang Peng, Ph.D.
Project Role:	Sr. Staff Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	5
Contribution to Project:	Expression of Twist and E47 and RUNX
Funding Support:	This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *NA*

QUAD CHARTS: *NA*

9. APPENDICES: *None*